EXHIBIT A

York (SUNY) at Stony Brook from 1974-1979. From 1979-1982, I was a postdoctoral research associate with Professor Harvey Ozer at Hunter College in

New York City. During that time in 1981, I received my doctoral degree from SUNY at Stony Brook where I published a thesis on T7 DNA replication. From 1982-1983, I continued as a postdoctoral research associate in the laboratory of Professor Martin Freundlich at SUNY at Stony Brook where I was intimately involved with sequencing of the ilvB locus of bacteriophage *E. coli* K12 using the Maxam-Gilbert technique. That investigation culminated in the publication "The ilvB locus of *E. coli* K12 is an operon encoding both subunits of acetohydroxyacid synthase," Nucleic Acids Research 13:3979-3993 (1985) coauthored by among others Philip Friden, myself James Donegan, and my advisor Dr. Freundlich. From 1983-1984, I continued as a post-doctoral research associate in Dr. Masayori Inouye's laboratory, also at SUNY-Stony Brook. While in Dr. Inouye's laboratory, I worked on developing methods for isolating species specific probes. A copy of my curriculum vitae is attached as Exhibit 1. A copy of my just-cited 1985 Nucleic Acids Research paper is attached as Exhibit 2.

3. During my academic and professional careers, I have become quite familiar with a number of techniques and methods in biotechnology, including cloning and vector technology, nucleic acid amplification and nucleic acid sequencing, to name just a few. Among my responsibilities at Enzo Diagnostics, Inc. have been the development of new nucleic acid amplification methods and new nucleic acid probe development based upon pathogenic agents, notably *N. gonorrhoeae* and various *Mycobacterium* species. While at Enzo, my group isolated and sequenced proprietary nucleic acid probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*.

4. I am familiar with the contents of U.S. Patent Application Serial No. 08/486,069, that was filed on June 7, 1995, based ultimately on a priority U.S. Patent Application No. 06/391,440 that was filed on June 23, 1982. I understand that among the presently pending claims in this application are claims 284-328 which are directed to a process for detecting a nucleic acid of interest, claims 329-336 directed to a process for determining the sequence of a nucleic acid of interest, claims 337-347 directed to a process for preparing a labeled oligo- or polynucleotide of interest, and claims 348-372 directed to a process for detecting the presence of an oligo- or polynucleotide of interest in a sequencing gel. A copy of the aforementioned claims 284-372 is attached as Exhibit 3.

5. I have read the Office Action dated January 6, 1998 that was issued in connection with this application. I understand that in this Office Action certain of the claims in this application were rejected for lack of an adequate written description, or new matter. The Examiner's position in part on the written description issue taken from the January 6, 1997 Office Action is as follows:

> Consideration of the disclosure as filed has failed to reveal support for newly submitted claims 339-341. It is noted that claims 79-82 and 90 disclosure some specific linkage groups but none of the same scope as newly submitted claims 339-341. The linkages of claims 339-341, 350, 351, 353, 354, 356-358 therefore are NEW MATTER. This rejection is necessitated by amendment.
>
> The optional template dependent or independent limitations of claims 346 and 363 have not been found as filed and are therefore NEW MATTER. This rejection is necessitated by amendment.
>
> The specific localization of modified nucleotides as given in instant claims 365-367 has also not been found as filed and is therefore NEW MATTER. This rejection is necessitated by amendment.
>
> The electrophoretic separating as given in instant claim 368 has also not been found as filed and is therefore NEW MATTER. This rejection is necessitated by amendment.
>
> Consideration of the disclosure as filed has also failed to reveal written description of sequencing gel practice as now given in instant claims 329 and 348 etc. This practice therefore is NEW MATTER. This rejection is necessitated by amendment.

6. I am making this Declaration in order to show that there is support in the specification for the written description of the claims at hand. Based upon my education and background, including my training and professional and academic experience, it is my opinion and conclusion that the specification for this application reasonably conveys that the inventors were in possession of the subject matter embraced by claims 329, 339-341, 346, 348, 350-351, 353-354, 356-358, 363 and 365-367 at the time the application was originally filed in 1982.

Sequencing Gel Practice

7. Claims 329 and 348 are directed to detection and sequence determination processes conducted in sequencing gels, respectively. Claim 329 recites three steps including incorporating one or more modified self-signaling or self-indicating

or self-detecting nucleotides into a nucleic acid fragment or fragments, separating the labeled fragments in a sequencing gel, and detecting the presence of each specific segment of the labeled nucleic acid fragments by means of the self-signaling or self-indicating or self-detecting modified nucleotide. Claim 348 recites five steps including the first step of providing one or more chemically modified nucleotides, a second step of incorporating such modified nucleotides into an oligo- or polynucleotide of interest, a third step of transferring the labeled oligo- or polynucleotide of interest to a sequencing gel, a fourth step of separating the labeled oligo- or polynucleotide of interest from other nucleic acids not of interest, and the fifth step of detecting directly or indirectly the presence of the labeled oligo- or polynucleotide of interest. It is my opinion and conclusion that the sequencing gel practice set forth in claims 329 and 348 is supported by the disclosure in the specification at page 84, second paragraph:

> This type of self-signaling molecule can be used to monitor any nucleic acid hybridization reaction. It is particularly important for detecting nucleic acids in gels (for example, **sequencing gels**).
> [bold added]

The basis for my opinion and conclusion is set forth in the paragraphs that now follow.

8. Sequencing gels and their use have been long accepted in the art, including at the time this application was originally filed in 1982. In fact, the very term "sequencing gel" and equivalent terminology such as gel sequencing of nucleic acids, DNA sequencing gels and nucleic acid sequencing gels, were well known and accepted in the art before 1982. The classic papers on nucleic acid sequencing were published by two groups of investigators headed by Walter Gilbert and Fred Sanger [Maxam and Gilbert, "A new method for sequencing DNA," Proc. Natl. Acad. Sci. (USA) 74:560-564 (February 1977); and Sanger et al., "DNA sequencing with chain-terminating inhibitors," Proc. Natl. Acad. Sci. (USA) 74:5463-5467 (December 1977)]. A copy of the Gilbert 1977 PNAS and Sanger 1977 PNAS papers are attached to this Declaration as Exhibits 4 and 5, respectively. Even before the 1977 publication of the now legendary papers by the Gilbert and Sanger groups, other more tedious methods for sequencing were available. These are described in a review paper by Sherman M. Weissman titled "Current Approaches to Analysis of the Nucleotide Sequence of DNA" [Analytical

Biochemistry 98:243-253 (1979)], a copy of which is attached as Exhibit 6. Among other older approaches to sequencing is the so-called "plus and minus" method also devised by Professor Sanger ["A Rapid Method for Determining Sequences in DNA by Primed Synthesis with DNA Polymerase," Journal of Molecular Biology 94:441-448 (1975)], copy of which is attached as Exhibit 7. Another method called "ribosubstitution" was published the year after Sanger's and Gilbert's 1977 PNAS papers and was developed by Wayne M. Barnes ["DNA Sequencing by Partial Ribosubstitution," Journal of Molecular Biology 119:83-99 (1978)], copy attached as Exhibit 8.[1]

In his famous 1977 PNAS paper, Professor Gilbert himself employed the term "sequencing gel" on page 563, right column, and continuing through page 564, left column:

> **Gel Samples.** All samples for *sequencing gels* are in 10 or 20 μl of 0.1 M NaOH/1 mM EDTA to which is added an equal volume of 10 M urea/0.65% xylene cyanol/0.05% bromphenol blue. Heat the sample at 90° for 15 sec, the layer on the gel.
>
> ***Sequencing Gels.*** These are commonly slabs 1.5 mm X 330 mm X 400 mm with 18 sample wells 10 mm deep and 13 mm wide separated by 3 mm (fitting on a 35.5 X 43 cm x-ray film). They are 20% (wt/vol) acrylamide (Bio-Rad)0.67% (wt/vol) methylene bisacrylamide/7 M urea/50 mM Tris-borate, pH 8.3/1 mM EDTA/3 mM ammonium persulfate, 300 ml of gel solution is polymerized with TEMED within 30 min (generally 50 μl of TEMED). Age the gel at least 10 hr before using it. Electrophorese with some heating (30-40°), to help keep the DNA denatured, between 800 and 1200 V. Load successively whenever the previous xylene cyanol has moved halfway down the gel. Bromphenol blue runs with 10-nucleotide-long fragments, xylene cyanol with 28. With three loadings at 0, 12, and 24 hr, a 1000-V run for 36 hr permits reading more than 100 bases. To sequence the first few bases from the labeled end, use a 25% acrylamide/0.83% bisacrylamide gel in the usual urea buffer and pre-electrophorese this gel for 2 hr at 1000 V.
>
> **Autoradiography.** Freeze the gel for autoradiography. . .
>
> [bold in original, italic & underline added]

Thus, from the very first Gilbert paper on sequencing, the term "sequencing gel" was not only christened, but described with such a great deal of specificity that

[1] Dr. Barnes' 1978 JMB paper is actually referenced in Dr. Sanger's 1977 PNAS paper on page 5463 (left column, 1st paragraph):

> . . . W. M. Barnes (*J. Mol. Biol.*, in press) has recently developed a third method, involving ribo-substitution, which has certain advantages over the plus and minus method, but this has not yet been extensively exploited.

the scientific reader would appreciate its meaning and its role in the sequencing process.

9. Several other authors and investigators followed suit by publishing articles that also used these terms.

A. For example, the very next year, 1978, at least two more publications appeared utilizing the term "sequencing gel." The first publication was the Barnes JMB paper discussed above and attached as Exhibit 8. Dr. Barnes describes a "sequencing gel" on page 93:

(f) Sequencing of cloned DNA

> øX174 is a convenient template for the experiments shown in Figures 1 to 3 because of its small size and easily available single-stranded DNA (the virion DNA). Many objects of DNA sequencing are likely to be cloned on a plasmid. Figure 5 illustrates the applicability of the partial ribosubstitution technique to DNA cloned on a plasmid. The sequence shown is from the promoter and genetic control region of the histidine operon of *Salmonella typhimurium*. This **sequencing gel** is presented as an example. Details of the histidine plasmid and its sequence will be published elsewhere when they are complete.
>
> Barnes, page 93, Exhibit 8, bold added

B. A second publication appeared that same year (1978). Winter and Brownlee published a paper with the very telling title "3' End labelling of RNA with ^{32}P suitable for rapid gel sequencing" [Nucleic Acids Research 5:3129-3138]. A copy of the Winter and Brownlee 1978 NAR paper is attached as Exhibit 9. In their "MATERIALS AND METHODS" section on pages 3132-3133, the authors provide the following description:

> (3) **Sequencing gel**. The conditions are essentially as described in Simonosits et al. [1] but were adapted for use with thin gels [18]. 1 µl aliquots of labelled tRNA were dried and digested in 2 µl as follows: 0.003 units Rnase T_1, 0.1 M Tris-HCl, 10 mM EDTA pH 7.5, 0°C, 2 min and 10 min; 2 pg RNase A, pH 7.5 buffer (as for T_1 RNase), 0°C, 5 min and 30 min; 0.4 units RNase U_2, 8.75 M urea, 20 mM sodium acetate, 2 mM EDTA, 50°C, 5 min; 0.0014 units RNase Phyl, 10 mM sodium acetate, 1 mM EDTA, pH 5.9, room temperature, 1½ min and 20 min; formamide containing 1 µl 1 M magnesium acetate per ml, 100°C, 30 min. For the ladder, traces of magnesium ion were added to the hot formamide to catalyse the degradation (R.H. Symons and G.P. Winter, unpublished). Partial

> alkali cleavage could have been used for the ladder since the mixture of cyclic phosphate and 2′ and 3′ phosphates so generated are not attached to the radioactively labelled fragments. With 5′ labelled sequences, however, the different mobilities of small oligonucleotides with cyclic and open phosphates introduces extra bands into the early portions of the ladder [1]. Time points were combined before running and a control of undigested $tRNA^{Phe}$ was included. Reactions were stopped by adding 2 µl of formamide dye mixture and heating at 100°C for 1 min. 2 µl of sample were applied to the thin gel which was electrophoresed at 1.6 kV for 4 h; the remaining 2 µl was applied after reheating and the gel electrophoresed for a further 2 h. The gel was exposed to preflashed film for five days [20].
>
> [bold added]

C. Three years after their seminal paper on DNA sequencing, Maxam and Gilbert published in Methods in Enzymology their paper titled "Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages [Maxam and Gilbert, Methods in Enzymology, Volume 65, Part I, Lawrence Grossman and Kivie Moldave, editors, Academic Press, New York, 1980, pages 497-701]. Indeed, the entire Section VIII of this Methods in Enzymology volume was devoted to "Nucleotide Sequencing Techniques," and it included in addition to Maxam and Gilbert's just-cited paper, nine other papers devoted to the subject. A copy of Section VIII from Volume 65, Part I of Methods of Enzymology, which section includes Maxam and Gilbert's paper, is attached as Exhibit 10. Maxam and Gilbert devote no less than six pages to a discussion of sequencing gels. See the subsection "Sequencing Gels" on pages 539-545. In addition to Maxam and Gilbert (1980), Andrew Smith ["DNA Sequence Analysis by Primed Synthesis," pages 560-580] described sequencing gels in a section titled "Interpretation of Sequencing Gels," pages 575-580 (Exhibit 10).

D. In 1980 two Russian investigators published a paper titled "Elimination of the Secondary Structure Effect in Gel Sequencing of Nucleic Acids" [Ambartsumyan and Mazo, FEBS Letters 114:265-268, copy attached as Exhibit 11]. In their introduction on page 265, the authors disclose that ". . . chemical modification that blocks the formation of secondary structure is of universal significance in gel-sequencing determination of the primary structure of nucleic acids." And in their results and discussion on page 268, left column, they write:

> As we have shown, the modification makes the secondary structure of tRNA and 5 S RNA unfold considerably, which may be

> attributed apparently to weakening of GC base pairs. As a result, 'compressions' caused by the secondary structure of RNA disappear from the **sequencing gel**.
>
> Therefore, the strategy for elimination of defects in electrophoretic separation of polyribonucleotides in [19] is applicable, as has been shown here, for DNA and may be of universal significance in **gel-sequencing** of nucleic acids.
>
> [bold added]

E. In the year before this application was filed, another German group published a paper reporting on "Improvements of DNA Sequencing Gels" [Garoff and Ansorge, Analytical Biochemistry 115:450-457 (1981), copy attached as Exhibit 12]. In their discussion on page 457, the authors disclose:

> The modification of the **DNA sequencing gel** described above (gel thickness 0.2 mm, covalent attachment of the gel to the glass, and the use of the thermostating plate) all contributed to an improved resolution of oligonucleotides in the gel. . .
>
> [bold added]

F. Yet another example dealing with nucleic acid sequencing gels was published a year later before this application was filed on June 23, 1982. In their paper titled "A semi-automated method for the reading of nucleic acid sequencing gels," Nucleic Acids Research 10:103-114 (1982), Gingeras et al. describe a collection of computer programs which permit automatic entering of nucleotide sequence data directly from an autoradiograph into a computer. A copy of Gingeras' 1982 NAR paper is attached as Exhibit 13.

G. The term "sequencing gel" was also accepted as a term of art in the first edition of Cold Spring Harbor's Molecular Cloning: A Laboratory Manual published in 1982 [edited by Tom Maniatis et al.], the same year as this application's filing. In Appendix A (Biochemical Techniques) of the first edition, a Protocol for Sequencing by the Maxam-Gilbert Technique is provided on page 475. On page 478 of that protocol, a recipe for "sequencing gels" is listed, including 20% acrylamide, urea mix, 5 X TBE and an 8% sequencing gel. A copy of the Maxam-Gilbert protocol from Maniatis' 1982 Manual is attached as Exhibit 14.

10. Even after the application's filing in 1982, the term "sequencing gel" continues to be well-accepted in the art even to this very year. Several scientific and technical dictionaries recognize the term "sequencing gel" including the

following:

A. A Dictionary of Genetic Engineering, Oliver and Ward, Cambridge University Press, Cambridge, 1985, page 100 [Exhibit 15]:

> **sequencing gel** A long polyacrylamide slab gel which has sufficient resolving power to separate single-stranded fragments of DNA or RNA which differ in length by only a single nucleotide. Electrophoresis is carried out at high voltage and with the gel in a vertical position. Urea is usually included in the gel mixture as a denaturing agent. This prevents internal base pairing within the single-stranded molecules and ensures that their relative speed of migration is solely dependent on their length. Such gels are used to separate the radioactively labelled products of, for example, the Maxam-Gilbert or the Sanger sequencing reactions.

B. Dictionary of Biochemistry and Molecular Biology, 2nd Edition, J. Stenesh, John Wiley & Sons, New York, 1989, page 437 [Exhibit 16]:

> **sequencing gel** A long, thin polyacrylamide gel slab used for nucleic acid sequencing.

C. Oxford Dictionary of Biochemistry and Molecular Biology, Smith et al., editors, Oxford University Press, New York, 1997, page 594 (Exhibit 17]:

> **sequencing gel** a polyacrylamide gel run to resolve oligonucleotides produced in a DNA sequencing procedure. *See* **chain-termination method, chemical cleavage method.** [bold & italic in original]

D. Dictionary of Plant Genetics and Molecular Biology, G. Miglani, The Food Products Press, New York, 1998, page 258 [Exhibit 18]:

> **sequencing gel:** A long, polyacrylamide salt gel that has sufficient resolving power to separate single-stranded fragments of DNA or RNA which differ in length by only a single nucleotide. Electrophoresis is carried out at high voltage and with the gel in a vertical position.

11. The term "sequencing gel" was also used quite liberally throughout a textbook that was devoted entirely to the topic of DNA sequencing and that was published the year after this application. As part of the series Laboratory Techniques in Biochemistry and Molecular Biology [DNA Sequencing, Elsevier, Amsterdam and New York, 1983, 384 pages], Hindley refers to the term "sequencing gel" no less than fifty-one (51) times on forty-five (45) different pages. A copy of these 45 pages from Hindley's DNA Sequencing book is

attached as Exhibit 19.

12. Based upon the foregoing publications, It is my opinion and conclusion that the use of the term "sequencing gels" in the specification of this application (page 84, second paragraph) reasonably conveys that the inventors were in possession of the sequencing practice set forth in claims 329 and 348.

Linkage Groups

13. With regard to the specific linkage groups of claims 339-341, 350-351, 353-354 and 356-358, it is my opinion and conclusion that the specification of this application fully supports the subject matter of these claims. Support for each of these claims in the specification is listed and described below.

Claims	Recitation	Support in Specification
339, 350 353, 356	**wherein said linkage group contains an amine**	**Page 11, 2nd full ¶** ("carbon-nitrogen bonds") **Page 11, 3rd full ¶** ("It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure –CH2–NH–, since such linkages are easily formed utilizing any of the well known amine modification reactions. Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxy-1-propyl) ether groups . . . Although these linkages are preferred, others can be used, including particularly olefinic linkage arms with other modifiable functionalities such as thiol, carboxylic acid, and expoxide functionalities. **Page 98, 1st ¶** ("Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached . . . via a chemical linkage or linkage arm as described in U.S. patent application Ser. No. 225,223, . . . The various linkages identifed in U.S. Ser. No. 225,223 aare applicable to and useful in the preparation of the special

		nucleotides of this invention.") **Originally filed claims 79-80, 90, 202-203**
340, 351 354, 357	**wherein said amine comprises a primary amine**	**Page 11, 3rd full ¶** **Page 13** ("to employ olefins with primary amine functional groups, such as allylamine (AA) or allyl-(-3-amino-2-hydroxy-1-propyl) ether (NAGE)") Page 16, schema ("allylamine") Page 18, 3rd ¶ ("Examples include .") Page 98, 1st ¶, *supra*.
341, 358	**wherein said linkage group does not substantially interfere with formation of the signalling moiety or detection of the detectable signal**	**Page 7, 2nd ¶** (Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic acid hybridization methodologies. To satisfy this criterion, it is preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or "linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.") **Page 11, 2nd ¶** ("The linkage or group joining moiety A to base B may include any of the well known bonds including carbon-carbon single bonds, or carbon-oxygen single bonds. However, it is generally preferred that the chemical linkage include an olefinic bond at the ∂-position relative to B. The presence of such an ∂-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double-helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide. **Page 96, 1st ¶** ("By way of summary . . . The chemical moiety Sig so

> attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide. . . The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide . . . and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation. **Page 97, 2nd ¶** (" . . . the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as to the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.)

Based upon the numerous citations listed above, it is my opinion and conclusion that the specific linkage groups of claims 339-341, 350-351, 353-354 and 356-358 is supported by the specification which reasonably conveys that the inventors were in possession of the subject matter of those claims at the time of their 1982 filing.

Template Dependent or Template Independent

14. Regarding the template dependent or independent limitations of claims 346 and 363, it is my opinion and conclusion that the specification supports the subject matter of these claims. It was known in the art that the incorporation of modified nucleotides, or for that matter, any nucleotide, can be carried out under template dependent or template independent reactions and conditions. Examples of template dependent incorporation and template independent incorporation reactions and methods are listed below, including citations to the specification that

describe them.

TEMPLATE DEPENDENT INCORPORATION

Example	Citation in Specification
nick translation	**Page 31, 1st full ¶** ("Modified nucleotides may be used in a method of gene mapping by *in situ* hybridization which circumvents the use of radioisotopes. This procedure takes advantage of a thymidine analogue containing biotin that can be incorporated enzymatically into DNA probes by nick translation.") **Page 32, 2nd ¶** (DNA probes were nick translated in the presence of Bio-dUTP . . .") **Page 67, Example XX** ("DNA was labeled with 5-substituted pyrimidine triphosphate by nick translating DNA in the presence of the appropriate triphosphate. . .") **Page 69, Example XXII** ("Phage T4 DNA and phage DNA were labeled by incorporation of H3-deoxyadenosine triphosphate into the DNA by nick translation . . .") **Add Page 70, last ¶** (". . . As previously indicated herein, nick translation is only one of a number of techniques and approaches possible for the production of the modified nucleic acids in accordance with this invention.") **Page 71, Example XXIII** ("Lambda DNA was nick translated . . .")
DNA polymerase	**Page 25, 1st ¶** ("These compounds can be made by enzymatic polymerization of appropriate nucleotides, especially nucleotide triphosphates in the presence of a nucleic acid template which directs synthesis under suitable conditions. . Illustrative enzymes include DNA polymerase I of E. coli, bacteriophage T4 DNA polymerase, DNA polymerases ∂ and ß from murine and human (HeLa) cells, DNA polymerase from Herpes simplex virus, . . ."

RNA polymerase	**Page 1, 6th & 7th lines from bottom** ("The biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro.) **Page 19, last five lines, through Page 20, line 9** ("Although biotinyl ribonucleoside triphosphates were found to function as substrates for the RNA polymerases . . . biotin-labeled RNA probes can be prepared enzymatically from DNA templates using E. coli or T7 RNA polymerases . . .") **Page 25, lines 10-13** (". . . RNA polymerase of E. coli, RNA polymerase of bacteriophage T7, eukaryotic RNA polymerase including HeLa cell RNA polymerase III, calf thymus polymerase II, and mouse cell RNA polymerase II.")

TEMPLATE INDEPENDENT INCORPORATION

Example	Citation in Specification
terminal transferase	**Page 56, Example III** ("Oligodeoxyribonucleotides were end-labeled using cytidine-5'-triphosphate and terminal transferase . . .") **Page 99, 2nd ¶** ("As indicated hereinabove, various techniques may be employed in the practices of this invention for the incorporation of the special nucleotides of this invention into DNA and related structures. One particularly useful technique . . . involves the utilization of terminal transferase for the addition of biotinated dUMP onto the 3' ends of a polypyrimidine or to single-stranded DNA. . .") **Page 100, 2nd ¶** ("Illustrative of the practices of this invention, biotinated dUTP was added to the 3' ends of d[pT]4 as well as single and double stranded DNA employing terminal transferase. . . The results . . . established that terminal transferase added biotinated dUMP to the 3' ends of a polypyrimidine.")

DNA ligation	**Page 77, Example XXXIV** ("A DNA probe was ligated to a synthetic DNA composed of repeated sequences of E. coli lac operator DNA. . .") **Page 60, Example IX** ("Ligation of poly dA:poly dT, biotinyl dU to oligodeoxyribonucleotides was accomplished as follows: . . .")
RNA ligation	**Page 20, lines 6-14** (". . biotin-labeled RNA probes can be prepared enzymatically . . . by 3' end-labelling methods using RNA ligase with compounds such as biotinyl-pCp. The AA- and NAGE-derivatives of UTP are, however, substrates for the eukaryotic RNA polymerases mentioned above.") **Page 25, 2nd ¶** (" . . . Moreover, the compounds such as pCp or pUp in which the base is biotinized can be added to existing molecules employing the enzyme RNA ligase.")
chemical modification	**Page 6, 1st full ¶** (" . . . Alternatively, nucleotides present in oligo- or polynucleotides may be modified using chemical methods.") **Page 25, 3rd ¶** ("Modified oligo- and polynucleotides can also be prepared by chemical modification of existing oligo- or polynucleotides using the approach described previously for modification of individual nucleotides.")

In view of the great many instances and examples in the specification where the chemically modified nucleotides are incorporated by means of template dependent or template independent reactions, it is my opinion that a reading of the specification reasonably conveys that the present inventors were in possession of the subject matter of claims 346 and 363 at the time this application was originally filed in 1982.

Localization of Modified Nucleotides

15. In regard to the specific localization of the modified nucleotides set forth in claims 365-367, it is my opinion and conclusion that the specification supports the subject matter claimed therein. All three claims depend from claim 348. Claim 365 recites "wherein the labeled oligo- or polynucleotide of interest prepared by said incorporating step comprises at least one internal modified nucleotide." Claim 366 recites "wherein the labeled oligo- or polynucleotide of interest prepared by said incorporating step comprises at least one external modified nucleotide." Claim 367 recites "wherein the labeled oligo- or polynucleotide of interest prepared by said incorporating step comprises at least one internal modified nucleotide and at least one external modified nucleotide."

A. With regard to incorporating at least one internal modified nucleotide as set forth in claim 365, the chemical structure on page 23 clearly shows an oligo- or polynucleotide having such an internal modified nucleotide. On page 24, penultimate paragraph, it is also disclosed that more than one modified nucleotide may be incorporated:

> It is also to be understood that the structure embraces more than one modified nucleotide present in the oligomer or polymer, for example, from two to thirty modified nucleotides. . . ."

And on page 27, second paragraph, it is also disclosed:

> . . . For example, pBR 322 DNA or λ DNA, nick translated to introduce approximately 10-100 biotin residues per kilobase, . . .

Moreover, the specification is replete with references and examples directed to the incorporation of modified nucleotides by nick translation. Nick translation so used would result in oligos- or polynucleotides each containing multiple modified nucleotides. See Page 67 (Example XX), Page 69 (Example XXII) and Page 71 (Example XXIII), cited above as support for template dependent incorporation of modified nucleotides into an oligo- or polynucleotide.

B. External modified nucleotides as set forth in claim 366 are disclosed in the specification by virtue of the numerous instances where terminal transferase

is disclosed or employed to add modified nucleotides to the 3' end of an oligo- or polynucleotide. See page 56 (Example III), page 99, 2nd paragraph, and page 100, 2nd paragraph, all cited above as support for template independent incorporation.

C. It is my opinion and conclusion that the subject matter of claim 367 to the effect that the "labeled oligo- or polynucleotide of interest prepared by said incorporating step comprises at least one internal modified nucleotide and at least one external modified nucleotide" is supported by the specification. As indicated above in Paragraph 14, means for incorporating modified nucleotides into oligo- and polynucleotides internally, such as by nick translation, and externally (terminally), such as by terminal transferase, are disclosed numerous times in the specification. As also indicated in the preceding Subparagraph 15A, the specification clearly discloses in the penultimate paragraph on page 24 that multiple modified nucleotides can be incorporated into an oligomer or polymer. These several disclosures in the specification, taken with the chemical formula on page 23 in which m or n can be zero, forms the basis of my opinion and conclusion that the subject matter of claim 367 is supported.

Based upon the numerous citations referenced above in Subparagraphs 15A, 15B and 15C, it is my opinion and conclusion that the subject matter of claims 365-367 reciting the specific location of the modified nucleotides in the oligo- or polynucleotide is supported by the specification which reasonably conveys that the inventors were in possession of that claimed subject matter.

Electrophoretic Separating Step

16. Concerning the electrophoretic separating step in claim 368, it is my opinion and conclusion that the specification fully supports the subject matter of this step and claim. At the time this application was filed in 1982, electrophoretic separation was an intrinsic step in sequencing gel practice. In several of the publications and definitions discussed above, electrophoretic separation or similar terminology is used to describe sequencing gels and sequencing gel practice.

A. For example, in the opening paragraph of Maxam and Gilbert's legendary 1977 PNAS paper, the authors disclose:

> We have developed a new technique for sequencing DNA molecules. The procedure determines the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenine, guanine, cytosine, or thymine with chemical agents. Partial cleavage of each base produces a nested set of radioactive fragments extending from the labeled end to each of the positions of that base. Polyacrylamide gel **electrophoresis** resolves these single-stranded fragments, their sizes reveal *in order* the points of breakage. The autoradiograph of a gel produced from four different chemical cleavages, each specific for a base in a sense we will describe, then shows a pattern of bands from which the sequence can be read directly. This method is limited only by the resolving power of the polyacrylamide gel. . .
>
> Maxam and Gilbert (1977), page 560, left column, 1st ¶, Exhibit 4. [bold added]

B. And in Sanger's equally legendary 1977 PNAS paper, his group provides the following description:

> **Principle of the Method.** Atkinson *et al.* (4) showed that the inhibitory activity of 2′,3′-dideoxythymidine triphosphate (ddTTP) on DNA polymerase I depends on its being incorporated into the growing oligonucleotide chain in the place of thymidylic acid (dT). Because the ddT contains no 3′-hydroxyl group, the chain cannot be extended further, so that termination occurs specifically at positions where dT should be incorporated. If a primer and template are incubated with DNA polymerase in the presence of a mixture of ddTTP and dTTP, as well as the other three deoxyribonucleoside triphosphates (one of which is labeled with ^{32}P), a mixture of fragments all having the same 5′ and with ddT residues at the 3′ ends is obtained. When this mixture is fractionated by **electrophoresis** on denaturing acrylamide gels the pattern of bands shows the distribution of dTs in the newly synthesized DNA. By using analogous terminators for the other nucleotides in separate incubations and running the samples in parallel on the gel, a pattern of bands is obtained from which the sequence can be read off as in the other rapid techniques mentioned above.
>
> Sanger et al. (1977), page 5463, left column, penultimate ¶,Exhibit 5. [bold added]

C. Wayne Barnes, Fred Sanger's post-doctoral fellow, was quick to place significance on the use of acrylamide gel electrophoresis for the separation of DNA fragments by size in his 1978 JMB paper [Exhibit 8]:

> . . . A key observation made by Sanger and his colleagues was that **electrophoresis on acrylamide gels** can resolve DNA molecules differing in length by a single nucleotide over the range of 20 to 140 nucleotides, if the molecules to be analysed all have the same sequence and share the same 5′ end (Barrell *et al.* 1976). Maxam

> and Gilbert (1977) use **similar high-resolution acrylamide gels** in a DNA sequencing method that uses the principle of end-labelling and base-specific partial chemical cleavage. . .
>
> Barnes (1978), page 83, Exhibit 8.
> [bold added]

D. Winter and Brownlee (1978) [Exhibit 9] intimately link electrophoresis with sequencing gel:

> (3) **Sequencing gel**. The conditions are essentially as described in Simonosits et al. [1] but were **adapted for use with thin gels** [18]. 1 µl aliquots of labelled tRNA were dried and digested in 2 µl as follows: 0.003 units RNase T_1, 0.1 M Tris-HCl, 10 mM EDTA pH 7.5, 0°C, 2 min and 10 min; 2 pg RNase A, pH 7.5 buffer (as for T_1 RNase), 0°C, 5 min and 30 min; 0.4 units RNase U_2, 8.75 M urea, 20 mM sodium acetate, 2 mM EDTA, 50°C, 5 min; 0.0014 units RNase Phyl, 10 mM sodium acetate, 1 mM EDTA, pH 5.9, room temperature, 1½ min and 20 min; formamide containing 1 µl 1 M magnesium acetate per ml, 100°C, 30 min. For the ladder, traces of magnesium ion were added to the hot formamide to catalyse the degradation (R.H. Symons and G.P. Winter, unpublished). Partial alkali cleavage could have been used for the ladder since the mixture of cyclic phosphate and 2′ and 3′ phosphates so generated are not attached to the radioactively labelled fragments. With 5′ labelled sequences, however, the different mobilities of small oligonucleotides with cyclic and open phosphates introduces extra bands into the early portions of the ladder [1]. Time points were combined before running and a control of undigested $tRNA^{Phe}$ was included. Reactions were stopped by adding 2 µl of formamide dye mixture and heating at 100°C for 1 min. 2 µl of **sample were applied to the thin gel which was electrophoresed** at 1.6 kV for 4 h; the remaining 2 µl was applied after reheating and the **gel electrophoresed** for a further 2 h. The gel was exposed to preflashed film for five days [20].
>
> [bold added]

E. In Maxam and Gilbert's 1980 Methods in Enzymology techniques paper [included in Exhibit 10], numerous instances are provided where electrophoresis or electrophoretic separation is routinely performed in sequencing gel practice. For example, on page 540, first full paragraph, the authors disclose:

> Gel sequencing methods have traditionally employed versions of a pH 8.3 polyacrylamide gel described by Peacock and Dingman[62] and adapted for small single-stranded DNA molecules by Maniatis *et al.*[63]

Later on page 543, Maxam and Gilbert provide a detailed procedure for loading and electrophoresing sequencing gels (*Procedure 18. Loading and Electrophoresing*

Sequencing Gels).

F. Garoff and Ansorge (1981) [Exhibit 12] give the following introduction to DNA sequencing techniques:

> The new and rapid DNA sequencing techniques involve the generation of a set of oligonucleotides, which have one end in common and the other end varying in length with a single nucleotide, and the subsequent separation of the oligonucleotides on denaturing polyacrylamide gels.
> Garoff and Ansorge (1981), page 430, right column, 1st ¶, Exhibit 12.

G. As described earlier in this Declaration, several scientific and technical dictionaries cite polyacrylamide gel in the definition of "sequencing gel." These include the following previously cited dictionaries and their definitions:

(i) A Dictionary of Genetic Engineering, Oliver and Ward, page 100 [Exhibit 15]:

> **sequencing gel** A long polyacrylamide slab gel which has sufficient resolving power to separate single-stranded fragments of DNA or RNA which differ in length by only a single nucleotide. **Electrophoresis** is carried out at high voltage and with the gel in a vertical position. Urea is usually included in the gel mixture as a denaturing agent. This prevents internal base pairing within the single-stranded molecules and ensures that their relative speed of migration is solely dependent on their length. Such gels are used to separate the radioactively labelled products of, for example, the Maxam-Gilbert or the Sanger sequencing reactions. [bold added]

(ii) Dictionary of Biochemistry and Molecular Biology, Stenesh, page 437 [Exhibit 16]:

> **sequencing gel** A long, thin polyacrylamide gel slab used for nucleic acid sequencing.

(iii). Oxford Dictionary of Biochemistry and Molecular Biology, Smith et al., editors, page 594 (Exhibit 17]:

> **sequencing gel** a polyacrylamide gel run to resolve oligonucleotides produced in a DNA sequencing procedure. *See* **chain-termination method, chemical cleavage method.** [bold & italic in original]

(iv) Dictionary of Plant Genetics and Molecular Biology, G. Miglani, page 258 [Exhibit 18]:

> **sequencing gel:** A long, polyacrylamide salt gel that has sufficient resolving power to separate single-stranded fragments of DNA or RNA which differ in length by only a single nucleotide. **Electrophoresis** is carried out at high voltage and with the gel in a vertical position.
>
> [bold added]

H. Hindley's book of DNA Sequencing, cited above in Paragraph 11 of my Declaration, addresses at the very outset the significance of gel electrophoresis in the development of DNA sequencing in the "Preliminary Remarks" of his Introduction (Chapter 1):

> 1.1 Preliminary Remarks
> The present art of DNA sequencing has its origins in a variety of different fields of nucleic acid enzymology and chemistry. Indeed as early as 1970 our knowledge and understanding of these fields was, in theory, sufficiently far advanced to anticipate the development of the modern rapid methods but two obstacles had first to be overcome to convert these ideas into reality. The first was the problem of separating the oligonucleotides, generated in the sequencing reactions, in a rapid convenient and reproducible manner and displaying them as an ordered set of fragments according to their chain length. While the technique of homochromatography, in which a random mixture of polynucleotides of all possible chain lengths is used to develop a chromatogram (Brownlee and Sanger, 1969), was an important step in this direction, it was through the development of gel electrophoretic techniques that this problem was finally solved. **All the methods to be described rely on the extraordinary resolving power of polyacrylamide gels** run under denaturing conditions to achieve the final separations; much effort has gone into perfecting such systems so as to optimise their resolving properties. . ."
>
> [Hindley, DNA Sequencing, page 1, Exhibit 20, bold added]

Based upon the foregoing documents referenced above in Subparagraphs 16A-16H and my own experience and knowledge at the time this application was filed in 1982, that it would have been understood that the practice and use of sequencing gels intrinsically involved electrophoretic separation as set forth in claim 368.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7/24/98
Date

James J. Donegan

* * * * * * *